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RESEARCH ARTICLE

Characterization of a novel allergenic protein from the octocoral *Scleronephthya gracillima* (Kuekenthal) that corresponds to a new GFP-like family named Akane

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Abstract

Certain marine organisms have been known to cause allergic reactions among occupational fishermen. We have previously reported that bronchial asthma among the workers engaged in spiny lobster fishing in Japan was caused by octocorals such as *Dendronephthya* sp. and *Scleronephthya gracillima* (previously named *Alcyonium gracillimum*).

Now we have found another octocoral, *Scleronephthya gracillima* (Kuekenthal), which causes the allergic disease in fishermen. The octocoral was characterized as a new green fluorescent protein (GFP)-like family. The new allergen has a molecular mass of 27 kDa in 1D and 2D SDS-PAGE under reduced conditions. The 27 kDa component was determined to be an allergen by western blotting, ECL immune staining method and absorption of patient sera with the antigen. Furthermore, the combination of analysis with LC-ESI-MS/MS and MASCOT search in the NCBInr database concluded the 27 kDa component had the sequence YPADI/LPDYFK, and that the 22 kDa component had the sequence QSFPEGFSWER, which both matched a GFP-like protein in *Acropora aculeus* and in *Montastraea annularis*. Further analysis by MALDI-TOF/MS/MS and MASCOT search in the NCBInr database of all 27 kDa eight spot components from 2D SDS-PAGE indicated that the sequence QSFPEGFSWER also matched as GFP-like protein in *Lobophyllia hemprichii* and *Scleractinia* sp. To our knowledge, this is the first report of the new allergenic protein that corresponds to a new GFP-like protein named Akane, and which has fluorescent emissions in the red and green part of the spectra at 628 nm and 508 nm, respectively.

KEYWORDS

allergenic protein, MALDI-TOF/MS/MS, new GFP-like protein named Akane, occupational allergy, octocoral

1 | INTRODUCTION

For decades, marine organisms have been considered as potential sources for exploitation as useful substances such as medicines and as biotechnical reagents like conotoxin,[1] nereis toxin[2] and others.[3,4]

Some marine organisms can cause severe food poisoning, such as puffer fish poisoning,[5,6] paralytic shellfish poisoning[7] and by cytotoxic constituents of octocoral *Dendronephthya* sp.[8] Moreover, they are also known to induce allergic reactions such as conjunctivitis, dermatitis and bronchial asthma among professional fishermen. Sea-squirt (*Styela plicata*), for example, is well known as an allergenic marine animal among workers engaged in oyster shucking, and sea-squirt oligosaccharides have been identified as allergens that induce allergic reactions such as conjunctivitis and bronchial asthma.[9-13] The structure of the sea-squirt oligosaccharide allergen epitopes

Abbreviations: CBB, Coomassie Brilliant Blue; CFP, cyan fluorescent protein; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; NMR, nuclear magnetic resonance; PBST, phosphate-buffered saline Tween; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RACE, rapid amplification of cDNA ends; UV, ultraviolet

was determined using two-dimensional (2D) nuclear magnetic resonance (NMR) methods and a molecular dynamics simulation method.[14]

A red octocoral, *Dendronephthya* sp., extract induced bronchial asthma in guinea pigs.[15] Recently, two different specimens of the red octocorals caught together with spiny lobsters in dragnet were reported to have caused allergic reactions among occupational fishermen of the spiny lobster fishing community along the Pacific coast of Miyazaki Prefecture, Japan.[16–18] These patients developed allergic reactions from octocoral including from *S. gracillima*, and had two or more of the following symptoms: nasal, dermal, respiratory and asthma. The major allergen in *Dendronephthya* sp. had a molecular mass of 53 kDa under reducing conditions,[17] while that of *Scleronephthya gracillima* (previously named *Alcyonium gracillimum*) was determined to be 17 kDa in mass.[18] During our recent survey for marine allergenic animals, newly collected *S. gracillima* specimens from the same area also had an enzyme-linked immunosorbent assay (ELISA) reaction-positive protein using pooled patients sera. It is possible that the presented materials from *S. gracillima* are of a different genotype than the former materials.[18–20] The purpose of the present study was to isolate new allergenic substances by monitoring using ELISAs and immunoblotting methods with pooled patients sera, and to determine the characteristics of new allergen. The allergen protein bands were analyzed by trypsin in-gel digestion and LC-ESI/MS/MS. The spectrum data were submitted for protein identification and database searching with the MASCOT search engine of the NCBInr database. The sequences of the allergenic protein bands matched that of a GFP-like protein. All amino acid sequences of the allergenic protein were determined using the cDNA method.

2 | EXPERIMENTAL

2.1 | Octocorals

The octocorals were caught on the Pacific coast of Miyazaki Prefecture, Japan by fishermen with the permission of the Miyazaki Prefecture. The octocoral was identified as *Scleronephthya gracillima* (Kukenthal).[20]

2.2 | Patient and control sera

The study was approved by the Institutional Review Board of Miyazaki-Higashi Hospital, Japan. Sera from patients with allergies were obtained from the Miyazaki-Higashi Hospital, and were then pooled. Patient sera were obtained from fishermen catching spiny lobsters along the Pacific coast of the Miyazaki Prefectures, who exhibited two or more of the following symptoms: conjunctivitis, rhinitis, dermatitis and bronchial asthma. The pooled patients' sera were combined and kept at −80°C. Negative control sera (control sera) were collected from healthy volunteers who did not exhibit allergy from octocoral and these were also pooled. All procedures were performed according to the ethical guidelines of Miyazaki-Higashi Hospital.

2.3 | Preparation of allergenic substances from *S. gracillima*

The octocoral *S. gracillima* was homogenized with 10 mM sodium phosphate buffer (PBS), pH 8.0. For the ammonium sulfate precipitation, ammonium sulfate was added to the supernatant to give a final concentration of 80% saturation. The precipitate was dialyzed exhaustively against distilled water.

2.4 | Allergen from the crude extract: ELISA, and purification

Gel filtration was conducted on a Superdex 75 column (2.5 × 45 cm, GE Healthcare BioSciences, Uppsala, Sweden) equilibrated with 10 mM PBS, pH 8.0. The reactivity to *S. gracillima* antigen-specific IgE in the pooled sera of patients was measured by ELISA.[21,22]

The specimen was then applied to a SP-Sepharose High Performance column (1.7 × 7 cm, GE Healthcare Bio-Sciences, Uppsala, Sweden), equilibrated with 10 mM acetate buffer pH 5.5. The column was eluted with same buffer at 5°C. Each fraction (3 ml) was analyzed using an ultraviolet (UV) light spectrometer at 280 nm wavelength. Unabsorbed allergen fractions concentrated using ultrafiltration USY-1 (Advantec Toyo, Japan) were combined. The specimen was then applied to a Q-Sepharose High Performance column (GE Healthcare Bio-Sciences, Uppsala, Sweden) which was equilibrated with 10 mM Tris–HCl buffer, pH 8.0 (THB). After being washed with the same buffer, the column was eluted stepwise with 10 mM THB containing 0.1, 0.15, 0.2, 0.25, 0.3, 0.4 or 0.5 M NaCl pH 8.0 at 5°C.

2.5 | Analysis of the allergen by SDS-PAGE and immunoblotting to detect IgE binding

One-dimensional (1D) SDS-PAGE was performed on a 15% acrylamide gel at 200 V for 75 min (Mini-Protean Cell, and Power Pac 300; Bio-Rad Laboratories).[23,24] Western blotting (Trans-Blot SD Semi-Dry Transfer Cell Power Pac 200, Bio-Rad Laboratories) was performed on a polyvinylidene difluoride (PVDF) membrane filter (Immobilon-psq, Millipore Corp, MA, USA) at 15 V for 30 min. To detect IgE binding, incubation was performed using the pooled patient sera or control sera for control reactions included 10% skimmed milk tp detect absorption [2000-fold with 10 mM phosphate-buffered saline Tween (PBST; 10 mM PBS and 3% Tween 20)]. Then the membranes were reacted with polyclonal goat anti-human IgE, horseradish peroxidase (HRP) conjugate, Cosmo Bioscience, BCA, USA) (15 000-fold with 10 mM PBST) which was used as the secondary antibody. For detection, a chemiluminescent reagent (ECL plus; Western Blotting Detection Reagents) (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used,[25,26] followed by exposure to a medical X-ray film (RX-U, Fujifilm, Kanagawa, Japan).

2.6 | Absorption method of patient sera with a crude antigen

Absorption of patient sera with crude antigen was performed as follows.[27] The patient sera were pre-incubated overnight at 4°C with crude extract from the *S. gracillima* in PBST. The pre-incubated sera

were used to react as a primary antibody. Then, analysis of the allergen was performed by SDS-PAGE and immune blotting, as before.

2.7 | Analysis of relative molecular mass

Using gel filtration chromatography, the molecular weight of the fraction (2.10) was analyzed.[28,29] The Superdex 200 10/30 GL gel (GE Healthcare, Buckinghamshire, UK) was equilibrated with 50 mM THB and 300 mM NaCl, pH 8.0, and then developed at 0.4 mg/ml.

2.8 | Trypsin-gel digestion and LC-ESI/MS/MS analysis

The 27 kDa, 22 kDa and 45 kDa components of fraction (2.10), which were detected by SDS-PAGE gel and Coomassie Brilliant Blue (CBB) staining, were each digested with trypsin solution (25 μg/μl trypsin in 50 mM sodium bicarbonate).

The digested peptide was then analyzed with LC-ESI/MS/MS LCQdecaXP (ThermoFisher Scientific K.K., Yokohama, Japan) in positive ion mode.[30–32] The spectral data were submitted for protein identification and database searching was performed with MASCOT ver1.9 (Matrix Science Ltd. London, UK) of the NCBInr database.[33] The searching parameters were as follows: all as taxonomy, enzyme of trypsin, 1 miss cleavage, variable modification of carbamidomethyl (C), oxidation (M), peptide tolerance of 2.0 Da, MS/MS tolerance of 0.8 Da, peptide charge of 1+, 2+ and 3+, and monoisotopic. Only significant hits, as defined by MASCOT probability analysis ($P < 0.05$), were accepted. 2D SDS-PAGE analysis had eight 27 kDa components. The digested peptide of the 27 kDa component were analyzed by MALDI-TOF/MS/MS[34–36] and by MASCOT search of the NCBInr database.

2.9 | The cloning of cDNAs corresponding to the allergenic protein of the *S. gracillima*

The *S. gracillima* cDNAs were cloned using the rapid amplification of cDNA ends (RACE) method. Poly(A) + RNA was purified using an oligodex-dT30 < Super > mRNA purification kit (TaKaRa Bio, Shiga, Japan) using total RNA prepared by the DNAiso plus kit (TaKaRa Bio). According to the manufacturer's protocol for the Smart RACE cDNA amplification kit (TaKaRa Bio USA, Mountain View, CA, USA), 1 μg of total RNA, cDNA for 5′-RACE and 3′-RACE were prepared. The cDNA of the Akane coding region was obtained by 3′-RACE using UPM primers. Amplified polymerase chain reaction (PCR) products were run on a 5% polyacrylamide gel, and then the band was dissected and purified using a QIAEX II DNA extraction kit (QIAGEN, Venlo, The Netherlands). The obtained fragments were cloned into the pCR4

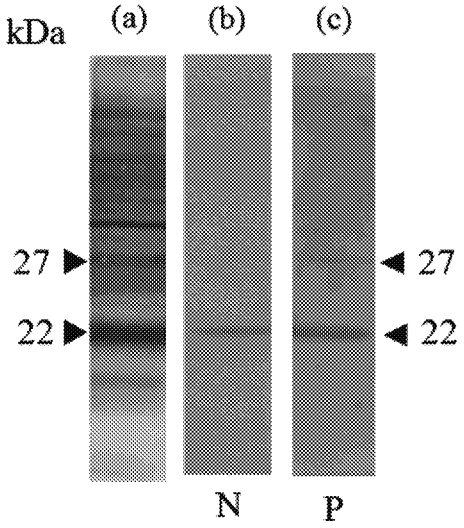

FIGURE 2 SDS-PAGE of crude protein extract from *Scleronephthya gracillima*, western blotting analysis and immunoblotting with control sera (N) and patient sera (P). (a) The crude protein was analysed by SDS-PAGE with a silver stain. (b) N: The 22 kDa component was found to react non-specifically with control sera by immunoblotting. (c) P: The 27 kDa component reacted only as an allergen with patient sera using immunoblotting, but the 22 kDa component reacted non-specifically

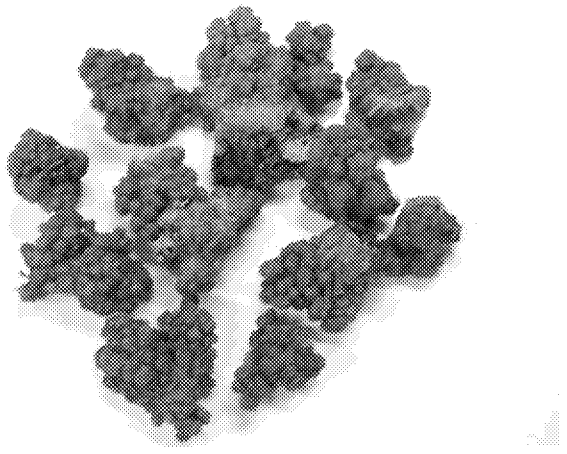

FIGURE 1 Appearance of fresh *Scleronephthya gracillima* (Kuekenthal). This soft coral is classified as Phylum Cridaria, Class Anthozoa, Subclass Octocorallia, Family Nephtheidae, *S. gracillima* (Kuekenthal). The colour of this octocoral is yellowish orange. It was collected from the Pacific coast of Miyazaki Prefecture, Japan

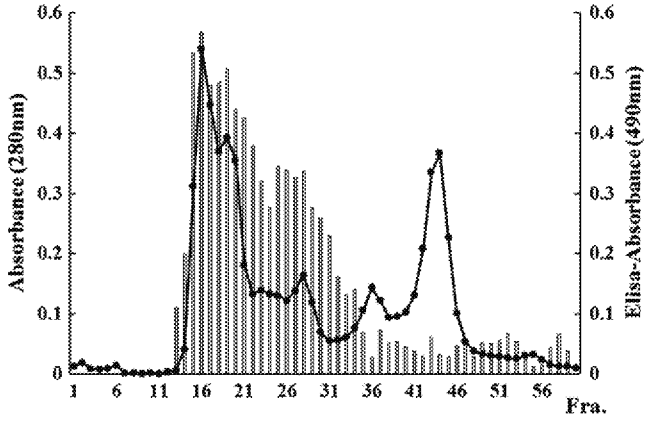

FIGURE 3 Fractionation of *S. gracillima* allergens using gel filtration on a Superdex 75 column. The extract was applied to a Superdex 75 column (25 × 45 cm) using 10 mM PBS pH 8.0. Fractions of 3 ml were analysed for UV absorbance at 280 nm (dot graph) and ELISA reaction absorbance at 490 nm (bar graph)

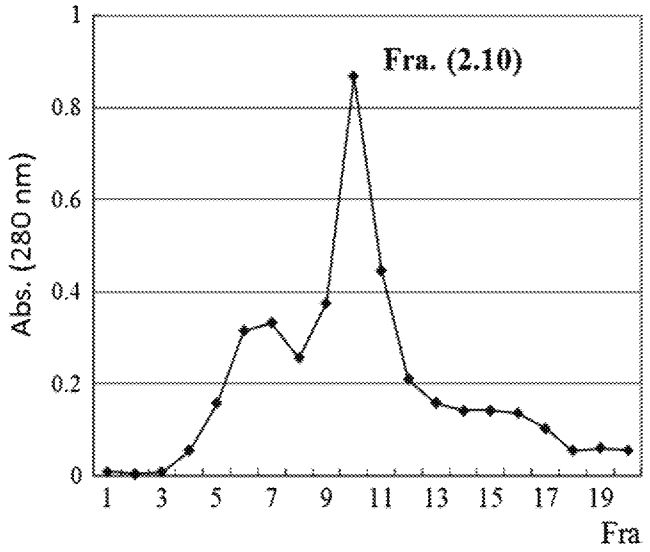

FIGURE 4 Anion exchange chromatographic fractions (using a Q-Sepharose high performance gel). Effluent buffer 10 nm Tris–HCl was used with 0.1 M NaCl pH 8.0, then all fractions were analysed for UV absorbance (280 nm). Fraction (2.10) was subjected to SDS-PAGE and immunoblotting analysis

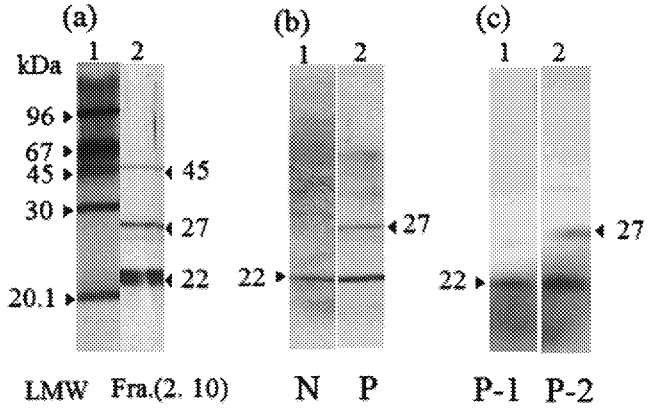

FIGURE 5 SDS-PAGE of a positive allergenic fraction. (a) Fraction (2.10) was analysed by SDS-PAGE and visualized with a silver staining kit. Lane 1: Molecular weight standards at 96, 67, 45, 30 and 20.1 kDa. Lane 2: Fraction (2.10) showing protein bands at 27, 22 and 45 kDa. (b) In immunoblotting analysis, both the 22 and 27 kDa components reacted with the pooled sera of patients (P) (lane 2): through non-specifically 22 kDa band with the control sera (N) (lane 1). (c) P-1 (lane 1): The absorbed serum reaction by western blotting analysis determined that the 22 kDa component was non-specific. P-2 (lane 2): In the non-absorbed serum, the 27 kDa and 22 kDa components appeared in fraction (2.10) of *S. gracillima*

vector (ThermoFisher Scientific, Waltham, MA, USA) and sequenced using the BigDye terminator cycle sequencing kit ver. 3.1 (ThermoFisher Scientific) on a ABI 3130 DNA analyzer (ThermoFisher Scientific). Deduced amino acid sequences were aligned by ClustalW and depicted by a BoxShade server.[37–39]

2.10 | Measurement of fluorescent emission of the fraction (2.10) component

The fraction (2.10) fluorescent protein components were extracted by THB buffer solution pH 8.0. Then, they were analyzed using a fluorescence spectrophotometer (Hitachi F-2500) with a quartz cell at excitation wavelengths 432 nm, 472 nm or 564 nm. The emission spectra were recorded with parameters as follows: scan speed set at 300 nm/min from 250 nm to 700 nm, both slit widths were set at 5.0 nm and photomultiplier voltage was set at 400 V for all measurements. The excitation spectra were recorded from emission wavelengths at 506 nm, 508 nm or 628 nm.

3 | RESULTS AND DISCUSSION

3.1 | Separation of the allergen from the octocoral *S. gracillima*

The octocoral *S. gracillima* (Figure 1) was found to react with pooled patient sera using ELISA. Some crude proteins from *S. gracillima* that were 22 kDa and 27 kDa components reacted with the pooled patient sera; a constituent at 22 kDa also reacted with the negative control sera using the SDS-PAGE and immunoblotting methods (Figure 2).

Crude protein was separated by gel filtration on a Superdex 75 column. Almost all obtained fractions reacted positively to some extent with the pooled sera of patients by ELISA, as shown in Figure 3. The results of the major allergen fractions (fractions 15–22) that had strong allergenic activity (Figure 3) were combined.

Fractions were concentrated using ultrafiltration USY-1 and then applied to a SP-Sepharose high performance column. Unabsorbed fractions were concentrated using ultrafiltration USY-1, and then applied to a Q-Sepharose high performance column to be fractionated. Fraction (2.10) was eluted from a Q-Sepharose high performance column by elution with 0.1 M NaCl in THB, pH 8.0 (Figure 4). Fraction (2.10) gave protein bands at 22, 27, and 45 kDa by SDS-PAGE (Figure 5a, lane 2). This fraction (2.10) was subjected to western blotting analysis and immunoblotting, which showed that the 22 and 27 kDa components reacted with pooled patient sera (Figure 5b, lane 2). However, the 22 kDa component also reacted non-specifically with the negative control sera (Figure 5b, lane 1). Furthermore, after absorption of the patient sera with *S. gracillima* crude antigen, the 27 kDa component disappeared (Figure 5c, lane 1). There was no absorption of the patient sera with crude antigen of *S. gracillima*, the 27 kDa component

TABLE 1 Proteome sequence results of 27 kDa, 22 kDa components from a 1D SDS gel of fra. (2.10). The 27 kDa component had sequence YPADI/LPDYFK, which matched the analogues of *Acropora aculeus* and scored 50, which is a cyan fluorescent protein (CFP)-like protein. The 22 kDa had sequence QSFPEGFSWER, which matched the analogues of *Montastraea annularis* and scored 65, which is a GFP-like protein

	MW (kDa)	Gene no.	Taxonomy match	Residue	Sequence
(1)	27 kDa	gi/ 51593122	*Acropora aculeus* (score 50) CFP-like protein	71–80	^{1}YPAD I/L PDYF^{80}K
(2)	22 kDa	gi/ 21303776	*Montastraea annularis* (score 65) GFP-like protein	81–91	^{81}QSFPEGFSWE^{91}R

appeared (Figure 5c, lane 2). These results suggest that the 27 kDa component protein was the only allergenic protein in *S. gracillima*.[27]

The 45 kDa component matched the FP-like protein from the proteome analysis (Table 1).

All components of fraction (2.10), 22 kDa, 27 kDa, 45 kDa, matched a GFP-like protein, but 27 kDa component only was an allergenic protein. It has been reported that the 43 kDa band is most likely due to the presence of a partially denatured GFP dimer.[39] The 45 kDa of fraction (2.10) may also be a denatured dimer.

The molecular weight of the protein in fraction (2.10) was analyzed at 67.4 kDa using gel filtration chromatography (figure not shown). The weight of the native protein Dend FP determined by gel filtration was 60 kDa as a dimer.[40] The molecular weight of the Akane protein is 67.4 kDa, which was considered as a dimer fluorescent protein such as in the *Dendronephthya* sp. octocoral.[40] and native DsRed was known as a dimer or a tetramer protein.[41,42] The eight components were determined as 27 kDa proteins, which have several pI values (Figure 6). It may be that one or more of the 27 kDa components bind with a phosphate group, and others may be bind to oligosaccharides.[43]

3.2 | Sequence of the 22, 27 kDa components from 1D SDS-PAGE gel, LC-ESI/MS/MS analysis and MASCOT search

The SDS-PAGE gel at 27 kDa had the sequence YPADI/LPDYFK and was matched as a CFP (GI number 51593122) in *Acropora aculeus* which was performed with a MASCOT ver. 1.9 search of the NCBInr database. Conversely, the sequence of trypsin-digested fragments of the 22 kDa component was determined to be QSFPEGFSWER, which had similarity to a GFP-like protein of *Montastraea annularis* (GenBank accession number AAK71332) using the same method. The 45 kDa

TABLE 2 Proteome sequence results of the Akane with eight components of 2D SDS-PAGE (Figure 7a). Components were labelled 1, 2, 3, 4, 5, 6, 7, 8 for the 27 kDa spots and all had sequence QSFPEGFSWER. They matched a fluorescent protein analogous to *Lobophyllia hemprichii*, and a fluorescent protein analogous to *Scleractinia* sp. The sequence QSFPEGFSWER was included in the total amino acid sequence of Akane (Figure 8)

Spot no. of 27 kDa components	Score	Analogue	Sequence
1	96	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
1	95	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R
2	95	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
2	94	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R
3	104	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
3	103	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R
4	104	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
4	103	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R
5	104	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
5	103	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R
6	104	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
6	103	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R
7	101	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
7	99	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R
8	101	*Lobophyllia hemprichii*	^{81}QSFPEGFSWE^{91}R
8	99	*Scleractinia* sp.	^{81}QSFPEGFSWE^{91}R

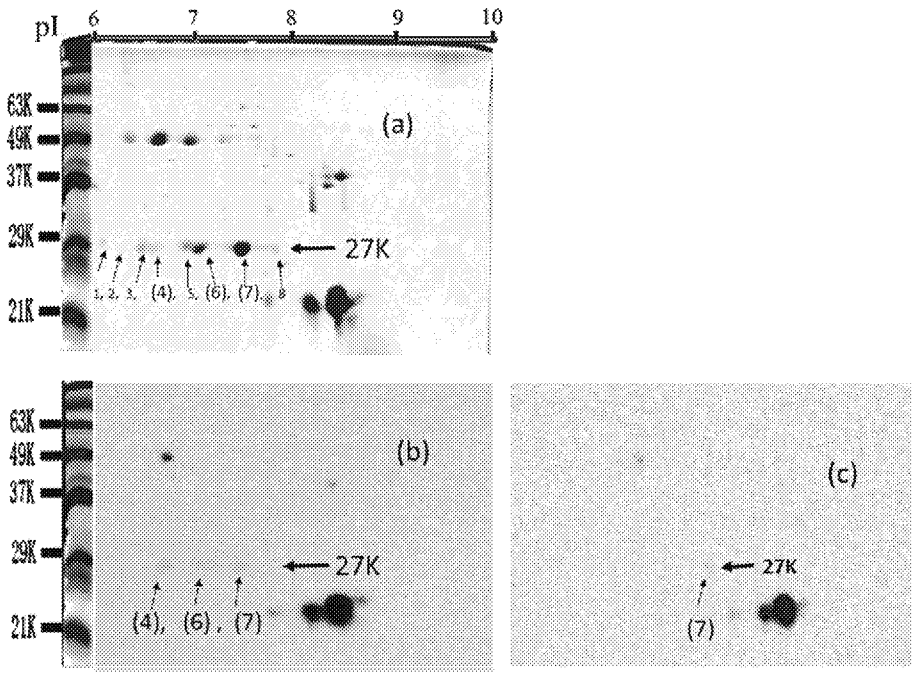

FIGURE 6 (a) The crude protein of the octocoral was analysed by 2D electrophoresis (pI 6 to 10) and all components were stained with CBB. All eight components were determined to be 27 kDa proteins (numbered 1 to 8). The octocoral was visualized using a combination of 2D-electrophoresis analysis and immunoblotting using patient sera (b) and control sera (c). (a) Molecular weight markers shown were 63, 49, 37, 29 and 21 kDa. (b) The three dots labelled as 4, 6 and 7 on the protein bands reacted with patient sera. (c) The dot labelled 7 reacted non-specifically with the control sera. The 27 kDa components labelled 4 and 6 were revealed as allergenic proteins

component had sequence YPADI/LPDYFK, and was also matched as a CFP (GI number 51593122) in *Acropora aculeus* (Table 1).

3.3 | Sequence from the 2D SDS-PAGE gel, Maldi-TOF/MS/MS analysis and MASCOT search

The crude protein from the octocoral was analyzed by 2D electrophoresis using a large gel that showed some components stained with CBB. The 27 kDa component appeared in the form of eight spots (numbered 1 to 8) in the pI range from 6 to 8 (Figure 6a).[42] They were determined by peptide mass fingerprinting analysis and MS/MS analysis against trypsin-digested peptides. Each of the eight components was digested, and these were determined as 27 kDa proteins. Using MALDI-TOF/MS/MS and MASCOT searches, all components contained the sequence QSFPEGFSWER as *Lobophyllia hemprichii* and *Scleractinia* sp. (Table 2).

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  1  MNPIKEDMKV KVYLEGNVNG
     HAFAIEGEGK  GNPLDGTQTM
 41  NLTVKEGAPL  PFSFDILTTS
     LHYGNRVFTK  YPADIPDYFK
 81  QSFPEGFSWE  RTMTYEDKGI
     CTVRSDISLQ  GDCFIQKVRF
121  HGINFPSNGP  VMQKKTLKWE
     PSTERMYVRD  GVLVGDINNA
161  LLLEGGGHYV  CDFKTTYKAK
     KVVQLPDYHF  VDIRIEILSH
201  DRDYNKVKLY  EHAVARHSLV
     PSQAR *
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FIGURE 7 The total amino acid sequence of 'Alkane' (225 residues) was determined by cloning cDNA analysis of *S. gracillima*. Sequence ^{62}H^{63}Y^{64}G is a chromophore of the fluorescent protein Akane. The sequences ^{71}YPADIPDYF^{80} and ^{81}QSFPEGFSWE^{91}R are shown also as the results of proteome analysis

Immunoblotting using patient sera and control sera were performed as shown in Figure 6(b,c). Three protein spots at 27 kDa reacted with patient sera as allergenic components numbered 4, 6 and 7 (Figure 6b). However, dot no. 7 also reacted non-specifically with the control sera (Figure 6c). Consequently, dot nos. 4 and 6 were revealed as allergenic proteins (Figure 6).

3.4 | Total amino acid sequence determination of Akane

Based on the amino acid sequences determined by LC-ESI/MS/MS, cDNAs were cloned using RACE. There were 225 amino acid residues in the amino acid sequences, and the chromophore, ^{62}H^{63}Y^{64}G, was found on Akane (Figure 7). It had sequence ^{62}H^{63}Y^{64}G, which was considered to be a chromophore of the fluorescent protein and had strong similarities to GFP-like families, such as *Dendronephthya* sp., Kaede, DsRed (Figure 8).[40,41,44,45] The sequences obtained by the MASCOT search, YPADI/LPDYFK, QSFPEGFSWER were all included in the total amino acid sequences of Akane (Figure 7).[46,47]

3.5 | The fluorescence emission and excitation spectra of the fraction (2.10)

Fraction (2.10) solution was pink, and cnidarian had a fluorescent protein. From the results of the proteome analysis, the component of fraction (2.10) was a fluorescent protein. In consequence, the molecular weight of the antigenic protein was a 27 kDa component, and it seemed to be the molecular size of GFP-like families.[40–42,44,45] The result of fluorescence measurements of fraction (2.10) showed some fluorescence emission wavelengths, such as 506, 508 and 628 nm, by an excitation wavelength at 432, 472 and 564 nm respectively (Figure 9). The Stokes shift of the Akane was about 64 nm, which was longer than other native red fluorescent proteins (Figure 10). The emission spectrum and the excitation spectrum are shown in Figure 9 (a–c). Akane had two fluorescent emissions, a green emission at 508 nm and a red emission at 628 nm. Two colour fluorescent emission

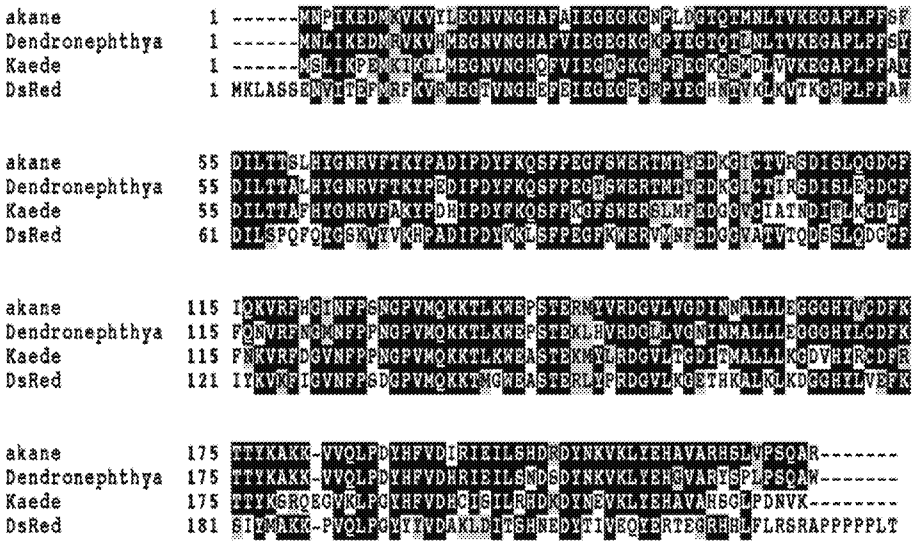

FIGURE 8 Total amino acid sequence of Akane (225 residues), *Dendronephthya* sp., Kaede and DsRed (Akane GenBank accession number LC193504)

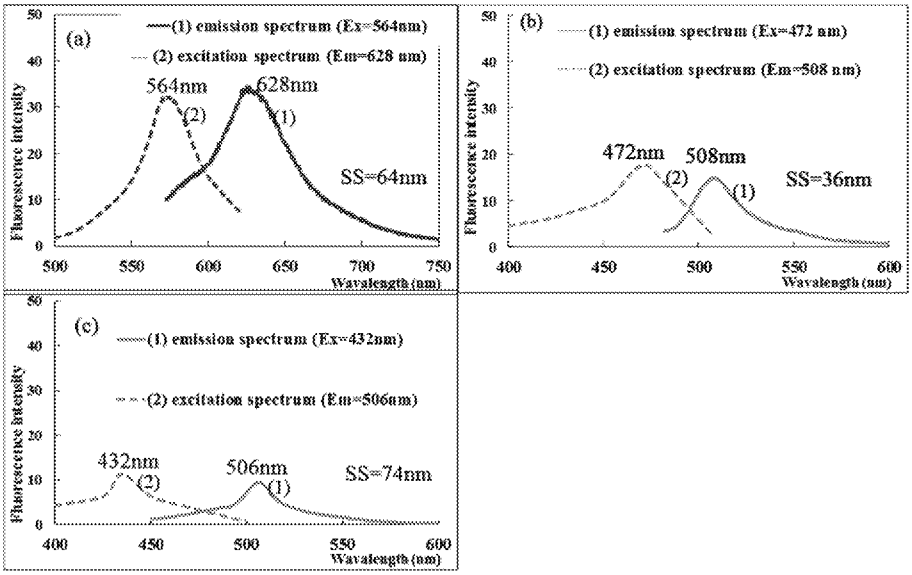

FIGURE 9 Excitation spectra and emission spectra of fraction (2.10) with three excitation wavelengths (Ex1 = 564 nm, Ex2 = 472 nm, Ex3 = 432 nm). (a) The emission spectrum (1) was gained by excitation wavelength 564 nm of fraction (2.10). The excitation spectrum was gained by emission wavelength 628 nm of fraction (2.10). (b, c) These were also gained using the same methodology. (a) Ex1 = 564 nm, Em = 628 nm, (b) Ex2 = 472 nm, Em = 508 nm, (c) Ex3 = 432 nm, Em = 506 nm. Stokes shift (SS): (a) 64 nm; (b) 36 nm; (c) 74 nm

GFP like protein (Native type)	Excitation wavelength (nm)	Emission wavelength (nm)	Storks shift (nm)
Akane	564	628	64
Dendronephtya sp.	560	575	15
Kaede	572	580	8
Ds Red2	563	582	19

FIGURE 10 Akane emits red fluorescence at 628 nm when excited at 564 nm. Akane has a long Stokes shift of about 64 nm. This value is longer than other native red fluorescent proteins such as *Dendronephthya* sp., Kaede and DsRed2

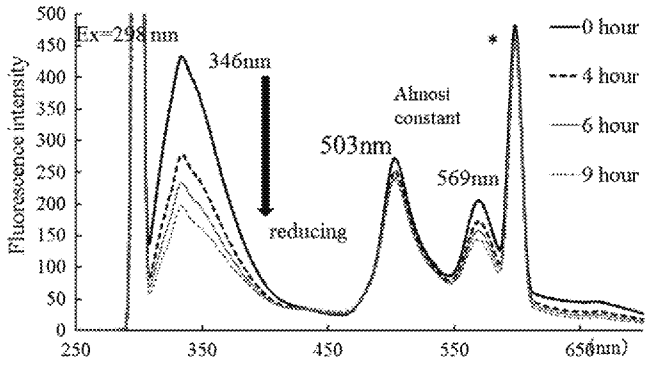

FIGURE 11 When *S. gracillima* fluorescent protein solution was excited at 298 nm, emissions at 346 nm, 503 nm, 569 nm were produced simultaneously. The 346 nm emission is considered to be from tryptophan (W) of the protein due to xenon lamp irradiation continuously for 0 to 9 h. The W emission at 346 nm was reduced, but the 503 and 569 nm emission intensities hardly changed. (*Secondary excitation wavelength)

for Kaede was considered because UV light induces green to red photoconversion on the chromophore.[40,44,45] However, for excitation at 298 nm, the two fluorescence intensities at 503 and 569 nm for Akane were not reduced by continuous 9 h irradiation with a 298 nm xenon lump light. The emission of tryptophan in the protein at 346 nm was reduced (Figure 11). In addition, mature chromophore red emission and immature chromophore green emission of *Discosoma* Red Fluorescent caused *cis-trans* isomerization or protonation-state changes in the chromophore HcRed.[48,49] Clarification of why Akane emits green and red fluorescence will need further research. The function and the structure of the fluorescent protein octocoral Akane will be reported in a future study.

4 | CONCLUSION

The molecular size of the 27 kDa allergenic protein of *S. gracillima* was much smaller than that of *D. nipponica* (53 kDa),[17] and larger than that of Takeda's material for *Scleronephthya gracillima* (previously named *Alcyonium gracillimum*) (17 kDa)[18]; all were analyzed under reduced conditions.

Further in this study, the allergenic protein from octocoral was confirmed to be a member of the GFP-like protein family using the LC-ESI/MS/MS method, MALDI-TOF/MS/MS analysis method and a MASCOT search. It was of interest that an allergen from *S. gracillima* was a GFP-like protein. This *S. gracillima* fluorescent protein also has some fluorescent emission, such as green at 503 nm and as red at 628 nm. Following excitation at 298 nm (Figure 11), two fluorescent intensities at 503 nm and 569 nm were not reduced by continuous irradiation of 9 h from a 298 nm xenon lump light, but tryptophan emission at 346 nm was reduced. The Akane chromophore HYG may not change from green to red by a β-elimination reaction following irradiation for Kaede, the *Dendronephthya* sp. Akane may have another mechanism as *cis-trans* isomerization.

In an excessive sunlight environment, fluorescent pigments from corals have a photoprotective system, and remove their thermal stress.[50–53] There is a self-protection system for the living body of corals when irradiated by ultraviolet light from sunlight.

The relationship between a fluorescent protein from an octocoral and an allergenic protein was revealed by this research. This report describes the first clear correlation between an allergenic protein and a fluorescent protein from octocorals.

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